

## RESEARCH ARTICLE

# Positive feedback promotes terrestrial emergence behaviour in an amphibious fish

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## ABSTRACT

Major ecological transitions such as the invasion of land by aquatic vertebrates may be facilitated by positive feedback between habitat choice and phenotypic plasticity. We used the amphibious fish *Kryptolebias marmoratus* to test the hypothesis that aquatic hypoxia, emergence behaviour and respiratory plasticity create this type of positive feedback loop that causes fish to spend increasing amounts of time on land. Terrestrially acclimated fish were more sensitive to aquatic hypoxia (emergence at higher  $P_{O_2}$ ) and were less hypoxia tolerant (shorter time to loss of equilibrium) relative to water-acclimated fish, which are necessary conditions for positive feedback. Next, we tested the prediction that exposure to aquatic hypoxia causes fish to emerge frequently, reduce gill surface area, and become less hypoxia tolerant. Indeed, fish exposed to severe aquatic hypoxia spent almost 50% of the time out of water and coverage of the gill lamellae by an inter-lamellar cell mass almost doubled. Fish exposed to aquatic hypoxia that could emerge from water were also more sensitive to subsequent acute aquatic hypoxia and were less hypoxia tolerant than normoxia-exposed controls. These results are opposite those of fish that cannot escape from aquatic hypoxia and presumably arise owing to plastic changes that occur during air exposure. Together, these results indicate that emergence behaviour begets further emergence behaviour, driven by gill remodelling which reduces aquatic respiratory function. This type of positive feedback may explain how amphibious behaviour has repeatedly evolved in fishes that occupy hypoxic aquatic habitats despite the associated challenges of life on land.

**KEY WORDS:** Hypoxia, Gill remodelling, Respiration, Phenotypic plasticity, Mangrove rivulus, *Kryptolebias marmoratus*

## INTRODUCTION

Spatial and temporal environmental heterogeneity creates a fundamental phenotype–environment mismatching problem for animals, as phenotypes well suited to one environment are often poorly suited to different environments (Agrawal, 2020). To minimize these functional trade-offs, animals that inhabit variable environments either adopt generalist ‘jack-of-all-trades’ phenotypes or are responsive to environmental conditions (Kassen, 2002; West-Eberhard, 2003). One form of responsiveness is phenotypic plasticity, the ability of an animal to modify its phenotype to

match prevailing conditions (Pfennig, 2021). Alternatively, animals may respond to variability by choosing to occupy the most favourable subset of available habitats (Edelaar et al., 2008). However, there is little empirical data about how plasticity and habitat choice interact to determine phenotypes in heterogeneous environments (Edelaar and Bolnick, 2019).

Phenotypic plasticity and habitat choice can sometimes negate or offset one another (Turko and Rossi, 2022). For example, in situations when organisms can always select optimal habitats, phenotypic plasticity is not needed to improve the environment–phenotype match (Edelaar et al., 2017; Schneider and Meyer, 2017). Similarly, phenotypically plastic animals are often habitat generalists, as phenotypes can easily be adjusted to suit various conditions (Asbury and Adolph, 2007; Manenti et al., 2013). However, plasticity and habitat choice can also reinforce one another via positive feedback, although empirical examples are rare (Dingemanse et al., 2010; Miner et al., 2005; West-Eberhard, 1989). In stream-living salamanders, individuals choose to inhabit fast-flowing riffles or slow-moving pools depending on morphological traits linked to swimming ability; phenotypic plasticity then reinforces these morphological differences between habitats (Lowe and Addis, 2019). Likewise, predatory ambush bugs choose to perch on flowers that are a similarly coloured to their own bodies, and then this match is improved via plastic changes to body colouration (Boyle and Start, 2020). These examples of habitat choice–plasticity feedbacks focus on spatially heterogeneous but temporally consistent environments. In many cases, however, environmental conditions vary in both space and time, but to date there has been little consideration of how plasticity and habitat choice interact in these scenarios.

In the face of temporal environmental change, animals must evaluate whether to remain and use plasticity to mitigate the phenotype–environment mismatch or instead move elsewhere (Hendry, 2016; Scheiner, 2016). If environmental changes are gradual, plasticity that produces a more ‘tolerant’ phenotype may be expected given that dispersal often has fitness costs (Edelaar et al., 2008). However, if the rate of environmental change is too rapid to be accommodated by plasticity or when there are limits to the expression of plasticity, animals instead may simply leave the changing environment. In these situations, movement to an alternative habitat may then cause plastic changes that enhance phenotype–environment matching in the new habitat. As a result, the longer an animal remains in the new habitat, the more it may become mismatched to the original habitat, making a return less likely. In this way, the existence of habitat choice–plasticity feedback may reduce the occurrence of habitat transitions but also increase their success.

One of the most dramatic environmental transitions experienced by any animal occurs when amphibious fishes move between water and land (Damsgaard et al., 2019; Ord and Cooke, 2016; Turko et al., 2021). The fundamental physical differences between aquatic

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and terrestrial habitats result in a suite of functional trade-offs (Dejours, 1988; Wright and Turko, 2016). For example, fishes in water benefit from a large gill surface area that enhances oxygen uptake and improves tolerance to hypoxia, but in terrestrial conditions, large gills may be a liability because of the potential for damage and evaporative water loss (Nilsson et al., 2012). This respiratory trade-off is especially problematic for amphibious fishes, as many species live in hypoxic aquatic habitats (Graham, 1997) and severe hypoxia is a common proximate reason why amphibious fishes leave water (e.g. Livingston et al., 2018; Mandic et al., 2009; Urbina et al., 2011; reviewed by Sayer, 2005). Once out of water, many amphibious fishes use alternative respiratory surfaces and gills may become nonfunctional (Randall, et al., 1981). Some amphibious fishes, such as the mangrove rivulus, *Kryptolebias marmoratus* (Ong et al., 2007), and bichir, *Polypterus senegalus* (Turko et al., 2019a), modulate this gill surface area trade-off by reversibly altering the size of the interlamellar cell mass (ILCM). In air, these fishes increase the ILCM, reducing overall functional gill surface area. However, upon the return to water, several days are required to decrease the ILCM and during this lag-time period, respiratory function in water is impaired (Turko et al., 2012). Thus, by moving to land to escape aquatic hypoxia, and then developing a slowly reversed terrestrial gill morphology that limits respiration in water, a feedback loop may emerge that promotes terrestrial habitat choice (Turko et al., 2018).

We experimentally tested the hypothesis that habitat choice (behavioural hypoxia avoidance) and morphological plasticity (gill remodelling) reinforce one another in a positive feedback loop using the amphibious mangrove rivulus as a model. These fish readily move between water and land and experience severely hypoxic aquatic habitats in the wild (Rossi et al., 2019; Taylor, 2012; Wright, 2012). We first tested the critical predictions that terrestrial acclimation should decrease aquatic hypoxia tolerance and increase the sensitivity of the hypoxic emergence response. Second, we tested the prediction that aquatic hypoxia exposure should increase the frequency of emergence behaviour, and in turn should decrease subsequent aquatic hypoxia tolerance owing to gill remodelling on land.

## MATERIALS AND METHODS

### Experimental animals

We used adult *Kryptolebias marmoratus* (Poey 1880) hermaphrodites (0.09±0.02 g; strain 50.91; Tatarenkov et al., 2010) bred in captivity at the University of Guelph. Fish were held individually in 120 ml plastic containers filled with 60 ml of brackish water (15‰, 25°C, 12 h:12 h light:dark cycle) and fed *Artemia* nauplii three times a week. To acclimate fish to terrestrial conditions (7 days), fish were placed on a filter paper substrate that was saturated with brackish water in standard housing containers as described previously (Ong et al., 2007). Control fish were held in identical containers filled with brackish water and were fasted for the 7-day acclimation period because mangrove rivulus cannot feed on land (Turko et al., 2019b; Wells et al., 2015). These acclimation conditions were used for each of the three experimental series described below. All experiments were approved by the University of Guelph Animal Care Committee (Animal Use Protocol 3891).

### Experimental protocol

#### Series 1: plasticity of hypoxia sensitivity and tolerance

To test how the sensitivity of the hypoxic emergence response varied between air- and water-acclimated fish (each  $n=10$ ), we measured the acute emergence response using established methods (Livingston et al., 2018; Regan et al., 2011). We placed individual

fish into the water section of a container composed of a lower section filled with water surrounded by an air-exposed platform, and allowed them to adjust (30 min) until they displayed normal behaviour (e.g. routine swimming without freezing or darting, no sign of hyperventilation), indicating that they were minimally stressed. In two cases, fish jumped out of water during this acclimation period; these fish were excluded from all analyses. After the acclimation period, we began to decrease the oxygen concentration by bubbling nitrogen gas from 21 to 0 kPa over 20 min. We measured water oxygen saturation using a fibre-optic oxygen probe (Witrox-4, Loligo Systems, Copenhagen, Denmark) and recorded the oxygen level at emergence.

To assess hypoxia tolerance, we measured time until loss of equilibrium (LOE) under severe hypoxia using standard methods (Regan et al., 2017). Briefly, we placed water- or air-acclimated fish (each  $n=6$ ) into mesh-sided chambers submerged in a 5-gallon (19 l) aquarium filled with brackish water to prevent the fish from reaching the air–water interface. The aquarium was covered with plastic film to minimize gas exchange with atmospheric air. Fish were allowed to recover for 30 min, after which we bubbled nitrogen gas to decrease the oxygen partial pressure ( $P_{O_2}$ ) from 21 to 0.5 kPa over 10 min, at which point it was held constant at 0.5 kPa for the remainder of the experiment (Regan and Richards, 2017; Regan et al., 2017). We recorded the time until fish lost equilibrium, defined as the point when the fish did not respond to a physical stimulus.

#### Series 2: emergence behaviour and hypoxia sensitivity

To test the prediction that hypoxia exposure should increase the frequency of emergence and subsequently decrease hypoxia tolerance, we exposed fish to one of three oxygen levels: normoxia (21 kPa oxygen;  $n=38$ ), hypoxia (2.1 kPa oxygen,  $n=39$ ) or extreme hypoxia (0.5 kPa;  $n=28$ ) for 7 days. Within each group, fish were acclimated to either water or air for 7 days immediately before the experiment began to determine whether differences in gill morphology caused by air versus water acclimation influenced emergence behaviour in hypoxia. Fish were held individually in containers like the ones used to assess the hypoxic emergence response (described above), except the lower, water-filled portion of the containers were mesh-sided and submerged in a larger water bath, allowing for free circulation of water. Oxygen saturation of the water bath was maintained with an oxygen control system (OxyCTRL, Loligo Systems) that automatically bubbled nitrogen gas into a header tank when oxygen concentrations exceeded the experimental setpoint.

At the beginning of these trials, the oxygen level in the water was first adjusted to the desired setpoint (normoxic, hypoxic or extremely hypoxic) and allowed to stabilize before fish were added. Fish were then gently placed directly into the aquatic section of the chamber, and video recording for subsequent measurement of emergence behaviour began immediately. Emergence behaviour was recorded during the 12 h of light per day, as there is no difference in emergence behaviour between day and night (Turko et al., 2011). For the duration of this habitat choice experiment, fish were not fed and great care was taken to minimize all human disturbance so as to not influence their behaviour. After this 7 day experiment, we measured the acute emergence response (total  $n=58$ ,  $n=9$ –10 per pre-acclimation/exposure combination) or time to LOE (total  $n=48$ ,  $n=7$ –9 per pre-acclimation/exposure combination) on random subsets of fish as described above.

To determine whether there was a relationship between emergence behaviour and gill morphology, a subsample of fish

(total  $n=30$ ,  $n=4-8$  per pre-acclimation/exposure combination) was euthanized with buffered tricaine methanesulfonate and processed for histology as described previously (Turko et al., 2011). We measured the proportion of ILCM coverage of 20 randomly selected lamellae spanning five filaments for each fish (Turko et al., 2014, 2018).

### Series 3: aquatic surface respiration

We noticed that some fish in the extreme hypoxia treatment never lost equilibrium despite remaining in water for lengths of time that exceeded the degree of tolerance measured in our LOE experiments, and hypothesized these fish were using aquatic surface respiration (Kramer and McClure, 1982). To test this hypothesis, we placed individual fish (air-acclimated  $n=4$ , water-acclimated  $n=5$ ) in a 5-gallon glass aquarium filled halfway with water and covered on all sides with the exception of a small viewing window. Fish were allowed to adjust for 15 min, and then we slowly introduced nitrogen gas to reduce oxygen levels at a constant rate ( $-0.4$  kPa  $O_2$   $\text{min}^{-1}$ ). Each trial was video recorded, and we quantified the proportion of time each individual fish used aquatic surface respiration (defined as when the mouth of the fish is within 1 mm of the surface of the water; Kramer and McClure, 1982) as a function of oxygen saturation in the water. We quantified the use of aquatic surface respiration in 10 min intervals, during which time  $P_{O_2}$  declined by  $\sim 4$  kPa. The mean  $P_{O_2}$  of each interval was used for statistical analysis. Scoring of aquatic surface respiration was done by a single observer (L.T.) who was not blinded to the acclimation condition of each fish.

### Statistical analysis

All statistics were calculated using R version 4.0.2. To compare oxygen saturation at emergence and time to LOE in water- versus air-acclimated fish, we used Student's  $t$ -tests (Series 1). To compare oxygen saturation at emergence, time to LOE and ILCM coverage among hypoxia exposure conditions (Series 2), we used two-way ANOVA, followed by Tukey's *post hoc* tests. To assess changes in emergence behaviour over time within each acclimation condition and hypoxia exposure condition, we used permutational two-way ANOVA (1,000,000 permutations, package *permuco*; <https://CRAN.R-project.org/package=permuco>) because our data did not meet assumptions of normality or homoscedasticity. Permutational ANOVA is similar to a parametric ANOVA, but rather than assuming a Gaussian distribution, the dataset is repeatedly randomized, and the proportion of randomly ordered datasets with greater treatment effects than the actual dataset is calculated. We used a series of two-way ANOVAs (one per oxygen tension) rather than a single three-way ANOVA because we found a marginal three-way interaction in our exploratory analysis ( $F=1.73$ ,  $P=0.056$ ) and because the key predictions made by the positive feedback hypothesis relate to patterns over time within each treatment rather than statistical comparison among treatments. Individual identity was included as a random factor in all models to account for repeated measurements over the 7-day trials. *Post hoc* comparisons were calculated using permutational  $t$ -tests (1,000,000 permutations) implemented using the *pairwise.perm.t.test* function (package *RVAideMemoire*; <https://CRAN.R-project.org/package=RVAideMemoire>), and  $P$ -values were adjusted for multiple comparisons using the Benjamini and Yekutieli correction, which controls the false discovery rate (Benjamini and Yekutieli, 2001). We used similar permutational ANOVA and *post hoc* permutation  $t$ -tests (with Benjamini and Yekutieli corrected  $P$ -values) to assess changes in the proportion of time fish used

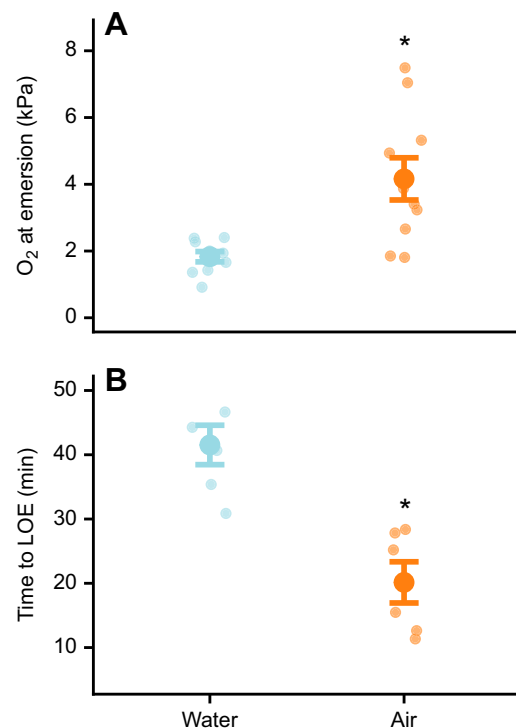
aquatic surface respiration during the induction of hypoxia (Series 3), as these data did not meet parametric assumptions of normality or homoscedasticity.

### RESULTS

Fish that were acclimated to air emerged at oxygen levels that were 2-fold higher than the oxygen levels that caused water-acclimated fish to emerge ( $t$ -test  $P=0.004$ ; Fig. 1A). Hypoxia tolerance (time to LOE) of air-acclimated fish was approximately half that of water-acclimated fish ( $t$ -test,  $P=0.0007$ ; Fig. 1B).

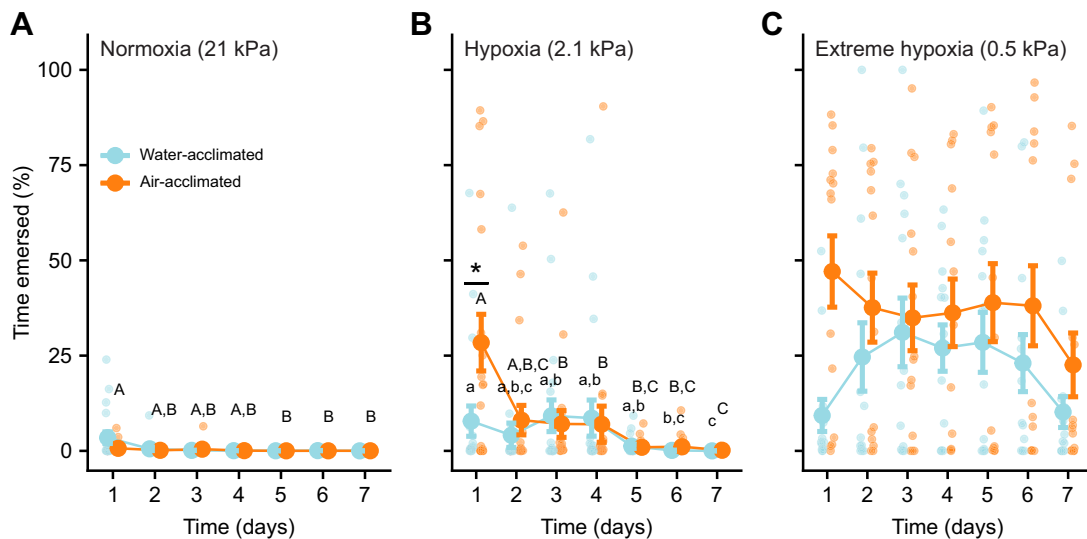
In our habitat choice experiment, exposure to aquatic hypoxia dramatically increased the amount of time fish spent out of water (Fig. 2). Fish in normoxia rarely emerged from water over the course of the trial, and emergence frequency in previously air-acclimated fish slightly but significantly decreased over the 7 day trial (time $\times$ acclimation  $P=0.0069$ ; Fig. 2A). In moderate hypoxia, emergence frequency similarly decreased over time, and previously air-acclimated fish initially spent significantly more time out of water than water-acclimated fish (time $\times$ acclimation  $P=0.0072$ ; Fig. 2B). In extreme hypoxia, fish regularly emerged from water ( $\sim 30\%$  of the time) but this did not depend on previous acclimation condition ( $P=0.12$ ) or time ( $P=0.057$ , interaction  $P=0.11$ ; Fig. 2C).

At the end of the habitat choice experiment, exposure to extreme aquatic hypoxia almost doubled the proportion of gill lamellae that were covered by an ILCM compared with normoxic or hypoxic conditions (two-way ANOVA,  $P=0.002$ ; Fig. 2A–D). Fish exposed to extreme hypoxia also had a significantly more sensitive hypoxic emergence response (two-way ANOVA,  $P<0.0001$ ; Fig. 3E). Finally, exposure to aquatic hypoxia decreased hypoxia tolerance



**Fig. 1. Sensitivity and tolerance of *Kryptolebias marmoratus* to aquatic hypoxia after acclimation to water or air.** (A) Water  $P_{O_2}$  at the point of emergence ( $n=10$  per treatment). (B) Time until loss of equilibrium (LOE) at water  $P_{O_2}$  of 0.5 kPa ( $n=6$  per treatment). Small points denote raw values (see Table S1), large points denote group means and error bars show standard error. An asterisk denotes significant difference between acclimation treatments ( $P<0.05$ ).





**Fig. 2. Habitat choice in *K. marmoratus* exposed to varying levels of aquatic hypoxia.** Proportion of time air- or water-acclimated fish spent on land during exposure to water of one of three oxygen pressures: (A) normoxia ( $n=38$ ), (B) hypoxia ( $n=39$ ) and (C) extreme hypoxia ( $n=28$ ). The first time point (day 1) represents data from the first 24 h of the experiment, as emergence was quantified beginning immediately after fish were placed in the choice arena. Small points denote raw values (see Table S1), large points denote group means and error bars show standard error. Different letters, when present, denote statistical differences within an acclimation group over time (lowercase letters show differences within water-acclimated fish, uppercase letters show differences within air-acclimated fish;  $P < 0.05$ ). An asterisk denotes significant difference between acclimation treatments at a single time point ( $P < 0.05$ ).

(time to LOE) by approximately 50% (two-way ANOVA,  $P < 0.00001$ ; Fig. 3F). In all cases, there was no significant effect of previous acclimation to air versus water (all  $P > 0.05$ ).

Mangrove rivulus significantly increased their use of aquatic surface respiration when oxygen tension fell below 10 kPa ( $P < 0.0005$ ; Fig. 4). At the lowest  $P_{O_2}$  we tested (1.5 kPa), fish used aquatic surface respiration for 95% of the recording period. This pattern of aquatic surface respiration was not affected by prior acclimation to air or water ( $P > 0.05$ ; Fig. 4).

## DISCUSSION

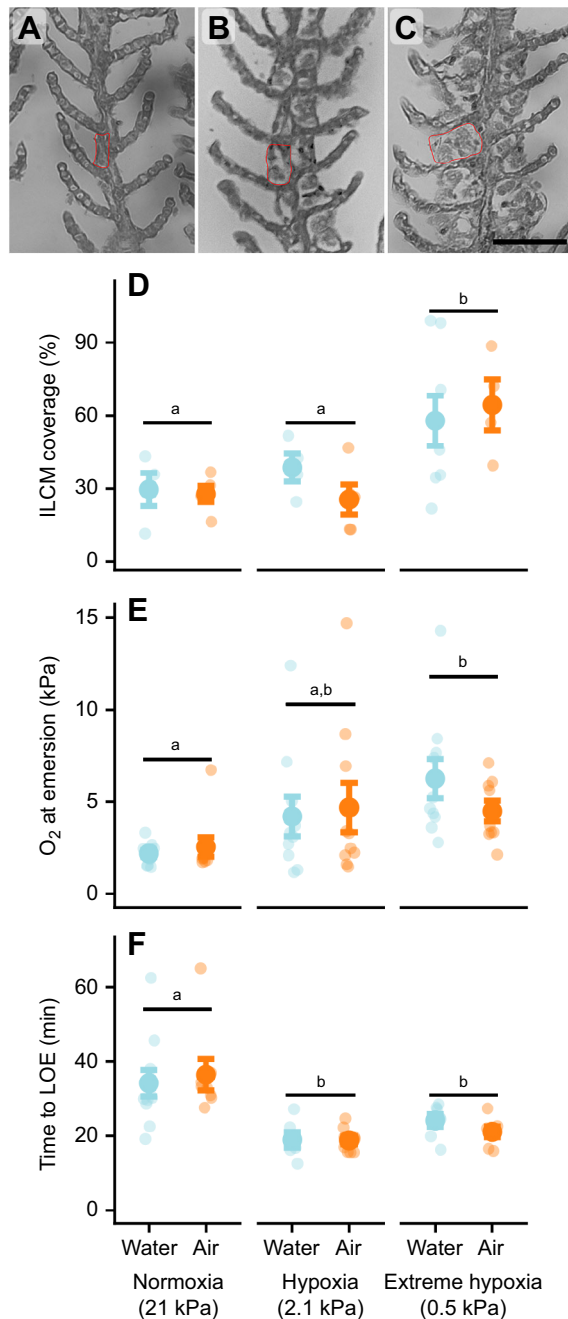
We found three lines of evidence consistent with the hypothesis that hypoxia-induced emergence behaviour can become self-reinforcing via positive feedback as gill remodelling on land decreases respiratory function in water and promotes emergence behaviour. First, air-acclimated fish emerged at higher oxygen levels relative to water-acclimated fish, indicating increased sensitivity to aquatic hypoxia. Second, air acclimation decreased aquatic hypoxia tolerance, increasing the reliance on avoidance behaviour for coping with harsh aquatic conditions. These are both necessary conditions for the establishment of a positive feedback loop. Third, after 7 days of exposure to hypoxic water with access to land, the sensitivity of the hypoxic emergence response increased while hypoxia tolerance decreased. Overall, our data indicate that emergence behaviour is an important strategy for coping with adverse aquatic conditions in this species, consistent with previous studies (Rossi et al., 2019; Turko et al., 2018). Importantly, our data also indicate that emergence behaviour makes it increasingly difficult for these fish to maintain homeostasis in hypoxic water. We suggest that this sort of positive feedback between plasticity and habitat choice has widespread potential to generate extreme phenotypes in animals. Furthermore, we speculate that if habitat choice–plasticity feedback results in genetic assimilation (Crispo, 2007; Pigliucci et al., 2006; Schneider and Meyer, 2017), this process may accelerate evolutionary habitat transitions such as the invasion of land by fishes.

## Requirements for positive feedback

There are two key elements necessary to generate positive feedback between habitat choice and phenotypic plasticity. First, expressions of plasticity that improve performance in one habitat must also decrease performance in alternative habitats (i.e. an antagonistic trade-off). Second, an animal must be capable of assessing its own phenotype and using this information to choose suitable habitats (i.e. matching habitat choice; Camacho et al., 2020). Our data indicate that both elements exist for mangrove rivulus moving between water and land, suggesting that the positive feedback hypothesis is plausible.

Mangrove rivulus face a suite of physiological trade-offs when moving between aquatic and terrestrial environments, the first requirement for positive feedback to occur. Many of these trade-offs involve the oxygen transport cascade. Gill remodelling on land may reduce evaporative water loss in air, but remodelling also decreases the capacity for oxygen uptake in water (Turko et al., 2012). Angiogenesis of cutaneous epidermal capillaries on land enhances aerial oxygen uptake (Blanchard et al., 2019), perhaps at the cost of oxygen loss to hypoxic water (Scott et al., 2017). The oxygen binding affinity of haemoglobin also increases in air, but in water this may impair oxygen offloading to metabolically active tissues (Tunnah et al., 2021; Turko et al., 2014). In the present study, terrestrial acclimation decreased overall aquatic hypoxia tolerance (i.e. time to LOE) by approximately 50%, indicating that the combined effects of these physiological trade-offs have a dramatic organism-level consequence.

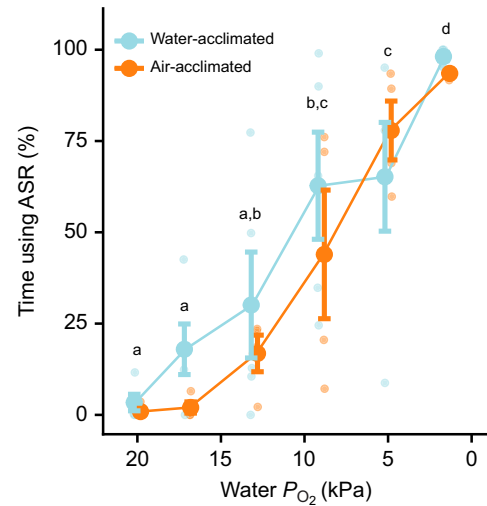
We also found evidence that habitat choice depends on physiological state (i.e. self-assessment), fulfilling the second requirement for positive feedback. When acutely challenged with progressive aquatic hypoxia, air-acclimated fish emerged at oxygen concentrations that were more than double that of water-acclimated fish. This result implies that mangrove rivulus can assess their physiological tolerance of aquatic hypoxia and make anticipatory decisions, as the concentrations of oxygen that elicited emergence were ~3- to 8-fold higher than the concentrations that these fish



**Fig. 3. Phenotypic plasticity of *K. marmoratus* after exposure to varying levels of aquatic hypoxia with access to terrestrial habitats.**

Representative micrographs of gills after exposure to (A) normoxia (21 kPa), (B) hypoxia (2.1 kPa) or (C) extreme hypoxia (0.5 kPa) for 7 days with access to terrestrial habitats. For illustrative purposes, a single inter-lamellar cell mass (ILCM) is outlined in red in each panel. Scale bar: 50  $\mu\text{m}$ . (D) Percentage of gill lamellae covered by an ILCM ( $n=4-8$  per group). (E) Water  $P_{\text{O}_2}$  at the point of emersion ( $n=9-10$  per group). (F) Time until loss of equilibrium at water  $P_{\text{O}_2}$  of 0.5 kPa ( $n=7-9$  per group). Small points denote raw values (see Table S1), large points denote group means and error bars show standard error. Different letters denote a significant difference between oxygen pressure treatment ( $P<0.05$ ).

could tolerate for 20–40 min in our LOE experiments. The mechanism(s) used for self-assessment of hypoxia sensitivity are not known, but previous work has found that the hypoxic emergence response of this species is regulated by oxygen-sensitive neuroepithelial cells (Regan et al., 2011). One



**Fig. 4. Use of aquatic surface respiration (ASR) by *K. marmoratus*.**

Points show the percentage of time individual fish (previously water- or air-acclimated) spent with the mouth within 1 mm of the surface of the water in response to decreasing oxygen concentrations ( $n=6$  per treatment; note the reversed x-axis). ASR was measured in 10-min intervals while  $P_{\text{O}_2}$  was continually decreasing ( $-0.4 \text{ kPa min}^{-1}$ ); plotted x-values represent the mean  $P_{\text{O}_2}$  of each of these intervals. Small points denote raw values (see Table S1), large points denote group means and error bars show standard error. Different letters denote overall statistical differences between levels of oxygen ( $P<0.05$ ).

possibility is that self-assessment occurs via internally oriented neuroepithelial cells that sense blood  $P_{\text{O}_2}$  (Perry et al., 2009), as impaired respiratory function in air-acclimated fish would presumably cause a faster decrease in blood  $P_{\text{O}_2}$  compared with water-acclimated fish. Understanding the mechanism of self-assessment is an exciting area for future research.

#### Establishment of an experimental positive feedback loop

Our habitat choice experiment provides further evidence for positive feedback between plasticity and habitat choice. We found that fish exposed to extreme aquatic hypoxia spent substantial amounts of time out of water (70-fold more than controls in normoxic water) and were more sensitive to aquatic hypoxia than fish exposed to normoxic water. Fish exposed to extreme hypoxia were also less hypoxia tolerant (i.e. time to LOE was reduced by  $\sim 40\%$ ) and had reduced functional gill surface area. Notably, these plastic changes are opposite to those typically observed with hypoxia acclimation in fishes (Perry et al., 2009; Richards, 2009). For example, when mangrove rivulus were prevented from leaving water during hypoxia acclimation, gill surface area increased and respiratory function improved (Turko et al., 2012). In contrast, the ILCM of fish exposed to extreme hypoxia covered the lamellae to a similar extent as previously measured in forcibly air-acclimated fish (Turko et al., 2012, 2018). Overall, our results indicate that when mangrove rivulus are given the choice between living in extremely hypoxic water or on land, the respiratory phenotype is shaped by the terrestrial habitat. Thus, habitat choice may be a key factor that shapes physiological tolerance of extreme environments.

Mangrove rivulus exposed to extreme aquatic hypoxia spent much more time out of water than fish in the other treatments, as predicted by the positive feedback hypothesis. However, contrary to the feedback hypothesis, emergence rates did not increase over the duration of the extreme hypoxia experiment despite the plastic expression of a terrestrial respiratory phenotype in these fish.

Emergence was also surprisingly infrequent in the moderate hypoxia treatment considering that acute exposure to this oxygen concentration elicited emergence in our first series of experiments. One explanation for both of these observations is that mangrove rivulus extensively used aquatic surface respiration (Chapman and McKenzie, 2009; Kramer and McClure, 1982) in combination with emergence to cope with aquatic hypoxia. Aquatic surface respiration allows fishes to selectively breathe from the relatively well-oxygenated layer of water at the air–water interface, thus reducing the severity of aquatic hypoxia. Our results nonetheless illustrate the interactive effects of habitat choice and physiological plasticity. Even subtle habitat choice, i.e. a shift up in the water column to use aquatic surface respiration, may have minimized the extent of aquatic hypoxia experienced by the fish and thus blunted plastic responses typical of hypoxia acclimation when fish cannot access the air–water interface. Reliance on aquatic surface respiration in our experiment may be a laboratory artefact. In the wild, mangrove rivulus under similarly hypoxic aquatic conditions spend ~90% of their time on land (Turko et al., 2018) and do not appear to frequently use aquatic surface respiration (A.J.T., personal observation), perhaps because this behaviour increases the risk of predation (Domenici et al., 2007; Kramer, 1983; Riesch et al., 2010). The terrestrial mangrove habitat provides many hiding opportunities amongst leaf litter and other debris, which we speculate may reduce the risk of predation compared with when fish use aquatic surface respiration.

## Perspectives

A fundamental goal of physiological ecology is understanding the processes that promote phenotype–environment matching (Bolnick and Otto, 2013; Botero et al., 2015; Edelaar et al., 2008). Two important mechanisms are behavioural habitat choice and phenotypic plasticity, but these mechanisms have often been studied in isolation. However, our results indicate that plasticity and matching habitat choice interact and can feed back on one another. Understanding these interactions can be important for interpreting patterns of phenotypic variation and predicting how animals will respond to changing environments. For example, our data showing that hypoxia-exposed mangrove rivulus have relatively low gill surface area and hypoxia tolerance cannot be explained without considering both plasticity and habitat choice in tandem.

Positive feedback moves systems away from equilibrium and as a result has been hypothesized to facilitate major evolutionary transitions (Crespi, 2004; Hendry, 2016) such as the invasion of land. On evolutionary timescales, phenotypic change coupled with matching habitat choice is thought to promote local adaptation and, potentially, speciation (Camacho et al., 2020; Jacob et al., 2017; Muñoz and Losos, 2018; Nicolaus and Edelaar, 2018). Our results indicate that analogous feedback between plasticity and habitat choice can cause phenotypic divergence within the lifetime of individuals. Given that expressions of plasticity can cause evolutionary change through genetic assimilation (Crispo, 2007; Pigliucci et al., 2006; Schneider and Meyer, 2017), we speculate that positive feedback between habitat choice and plasticity may be an important first step in generating similar evolutionary feedback between these processes. For example, populations of mangrove rivulus that inhabit moderately versus severely hypoxic habitats should differ in emergence rates and thus respiratory phenotype. If these environmental differences persist, evolutionary processes (e.g. genetic assimilation and/or altered selective environment) may reinforce the phenotypic divergence between populations. Over

time, this mechanism may therefore promote a major ecological transition by producing terrestrially adapted fishes that have poor aquatic performance owing to respiratory trade-offs.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: L.R.T., P.A.W., A.J.T.; Methodology: L.R.T., A.J.T.; Validation: L.R.T., P.A.W., A.J.T.; Formal analysis: L.R.T., A.J.T.; Investigation: L.R.T.; Resources: P.A.W.; Data curation: L.R.T., A.J.T.; Writing – original draft: L.R.T.; Writing – review & editing: L.R.T., P.A.W., A.J.T.; Visualization: L.R.T., P.A.W., A.J.T.; Supervision: P.A.W., A.J.T.; Project administration: P.A.W.; Funding acquisition: P.A.W.

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## References

- Agrawal, A. A. (2020). A scale-dependent framework for trade-offs, syndromes, and specialization in organismal biology. *Ecology* **101**, e02924. doi:10.1002/ecy.2924
- Asbury, D. A. and Adolph, S. C. (2007). Behavioural plasticity in an ecological generalist: microhabitat use by western fence lizards. *Evol. Ecol. Res.* **9**, 801–815.
- Benjamini, Y. and Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. *Ann. Statist.* **29**, 1165–1188. doi:10.1214/aos/1013699998
- Blanchard, T. S., Whitehead, A., Dong, Y. W. and Wright, P. A. (2019). Phenotypic flexibility in respiratory traits is associated with improved aerial respiration in an amphibious fish out of water. *J. Exp. Biol.* **222**, jeb186486. doi:10.1242/jeb.186486
- Bolnick, D. I. and Otto, S. P. (2013). The magnitude of local adaptation under genotype-dependent dispersal. *Ecol. Evol.* **3**, 4722–4735. doi:10.1002/ece3.850
- Botero, C. A., Weissing, F. J., Wright, J. and Rubenstein, D. R. (2015). Evolutionary tipping points in the capacity to adapt to environmental change. *Proc. Natl. Acad. Sci. USA* **112**, 184–189. doi:10.1073/pnas.1408589111
- Boyle, J. and Start, D. (2020). Plasticity and habitat choice match colour to function in an ambush bug. *Funct. Ecol.* **34**, 822–829. doi:10.1111/1365-2435.13528
- Camacho, C., Sanabria-Fernández, A., Baños-Villalba, A. and Edelaar, P. (2020). Experimental evidence that matching habitat choice drives local adaptation in a wild population: matching habitat choice in the wild. *Proc. R. Soc. B Biol. Sci.* **287**, 20200721. doi:10.1098/rspb.2020.0721
- Chapman, L. J. and McKenzie, D. J. (2009). Behavioral responses and ecological consequences. *Fish Physiol.* **27**, 25–77. doi:10.1016/S1546-5098(08)00002-2
- Crespi, B. J. (2004). Vicious circles: positive feedback in major evolutionary and ecological transitions. *Trends Ecol. Evol.* **19**, 627–633. doi:10.1016/j.tree.2004.10.001
- Crispo, E. (2007). The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61**, 2469–2479. doi:10.1111/j.1558-5646.2007.00203.x
- Damsgaard, C., Baliga, V. B., Bates, E., Burggren, W., McKenzie, D. J., Taylor, E. and Wright, P. A. (2019). Evolutionary and cardio-respiratory physiology of air-breathing and amphibious fishes. *Acta Physiol.* **228**, e13406. doi:10.1111/apha.13406
- Dejours, P. (1988). *Respiration in Water and Air: Adaptations-Regulations-Evolution*. Amsterdam: Elsevier Science Ltd.
- Dingemanse, N. J., Kazem, A. J. N., Réale, D. and Wright, J. (2010). Behavioural reaction norms: animal personality meets individual plasticity. *Trends Ecol. Evol.* **25**, 81–89. doi:10.1016/j.tree.2009.07.013
- Domenici, P., Lefrançois, C. and Shingles, A. (2007). Hypoxia and the antipredator behaviours of fishes. *Philos. Trans. R. Soc. B Biol. Sci.* **362**, 2105–2121. doi:10.1098/rstb.2007.2103
- Edelaar, P. and Bolnick, D. I. (2019). Appreciating the multiple processes increasing individual or population fitness. *Trends Ecol. Evol.* **34**, 435–446. doi:10.1016/j.tree.2019.02.001
- Edelaar, P., Siepielski, A. M. and Clobert, J. (2008). Matching habitat choice causes directed gene flow: a neglected dimension in evolution and ecology. *Evolution* **62**, 2462–2472. doi:10.1111/j.1558-5646.2008.00459.x
- Edelaar, P., Jovani, R. and Gomez-Mestre, I. (2017). Should I change or should I go? Phenotypic plasticity and matching habitat choice in the adaptation to environmental heterogeneity. *Am. Nat.* **190**, 506–520. doi:10.1086/693345
- Graham, J. B. (1997). *Air-Breathing Fishes: Evolution, Diversity, and Adaptation*. Academic Press.



- Hendry, A. P. (2016). Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. *J. Hered* **107**, 25–41. doi:10.1093/jhered/esv060
- Jacob, S., Legrand, D., Chaîne, A. S., Bonte, D., Schtickzelle, N., Huet, M. and Clobert, J. (2017). Gene flow favours local adaptation under habitat choice in ciliate microcosms. *Nat. Ecol. Evol.* **1**, 1407–1409. doi:10.1038/s41559-017-0269-5
- Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *J. Evol. Biol.* **15**, 173–190. doi:10.1046/j.1420-9101.2002.00377.x
- Kramer, D. L. (1983). The evolutionary ecology of respiratory mode in fishes: an analysis based on the costs of breathing. *Environ. Biol. Fish.* **9**, 145–158. doi:10.1007/BF00690859
- Kramer, D. L. and McClure, M. (1982). Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. *Environ. Biol. Fish.* **71**, 47–55. doi:10.1007/BF00011822
- Livingston, M. D., Bhargava, V. V., Turko, A. J., Wilson, J. M. and Wright, P. A. (2018). Widespread use of emersion and cutaneous ammonia excretion in aplocheiloid killifishes. *Proc. R. Soc. B* **285**, 20181496. doi:10.1098/rspb.2018.1496
- Lowe, W. H. and Addis, B. R. (2019). Matching habitat choice and plasticity contribute to phenotype–environment covariation in a stream salamander. *Ecology* **100**, e02661. doi: 10.1002/ecy.2661
- Mandic, M., Sloman, K. A. and Richards, J. G. (2009). Escaping to the surface: a phylogenetically independent analysis of hypoxia-induced respiratory behaviors in sculpins. *Physiol. Biochem. Zool.* **82**, 730–738. doi:10.1086/605932
- Manenti, R., Denoël, M. and Ficetola, G. F. (2013). Foraging plasticity favours adaptation to new habitats in fire salamanders. *Anim. Behav.* **86**, 375–382. doi:10.1016/j.anbehav.2013.05.028
- Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K. and Relyea, R. A. (2005). Ecological consequences of phenotypic plasticity. *Trends Ecol. Evol.* **20**, 685–692. doi:10.1016/j.tree.2005.08.002
- Muñoz, M. M. and Losos, J. B. (2018). Thermoregulatory behavior simultaneously promotes and forestalls evolution in a tropical lizard. *Am. Nat.* **191**, E15–E26. doi:10.1086/694779
- Nicolaus, M. and Edelaar, P. (2018). Comparing the consequences of natural selection, adaptive phenotypic plasticity, and matching habitat choice for phenotype–environment matching, population genetic structure, and reproductive isolation in meta-populations. *Ecol. Evol.* **8**, 3815–3827. doi:10.1002/ece3.3816
- Nilsson, G. E., Dymowska, A. and Stecyk, J. A. W. (2012). New insights into the plasticity of gill structure. *Respir. Physiol. Neurobiol.* **184**, 214–222. doi:10.1016/j.resp.2012.07.012
- Ong, K. J., Stevens, E. D. and Wright, P. A. (2007). Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure. *J. Exp. Biol.* **210**, 1109–1115. doi:10.1242/jeb.002238
- Ord, T. J. and Cooke, G. M. (2016). Repeated evolution of amphibious behavior in fish and its implications for the colonization of novel environments. *Evolution* **70**, 1747–1759. doi:10.1111/evo.12971
- Perry, S. F., Jonz, M. G. and Gilmour, K. M. (2009). Chapter 5 oxygen sensing and the hypoxic ventilatory response. *Fish Physiol.* **27**, 193–253. doi:10.1016/S1546-5098(08)00005-8
- Pfennig, D. W. (2021). *Phenotypic Plasticity and Evolution: Causes, Consequences, Controversies*. CRC Press.
- Pigliucci, M., Murren, C. J. and Schlichting, C. D. (2006). Phenotypic plasticity and evolution by genetic assimilation. *J. Exp. Biol.* **209**, 2362–2367. doi:10.1242/jeb.02070
- Randall, D. J., Burggren, W. W., Farrell, A. P. and Haswell, M. S. (1981). The Evolution of Air Breathing in Vertebrates. *Q. Rev. Biol.* **56**, 485–486. doi:10.1086/412496
- Regan, M. D. and Richards, J. G. (2017). Rates of hypoxia induction alter mechanisms of O<sub>2</sub> uptake and the critical O<sub>2</sub> tension of goldfish. *J. Exp. Biol.* **220**, 2536–2544. doi:10.1242/jeb.154948
- Regan, K. S., Jonz, M. G. and Wright, P. A. (2011). Neuroepithelial cells and the hypoxia emersion response in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **214**, 2560–2568. doi:10.1242/jeb.056333
- Regan, M. D., Gill, I. S. and Richards, J. G. (2017). Metabolic depression and the evolution of hypoxia tolerance in threespine stickleback, *Gasterosteus aculeatus*. *Biol. Lett.* **13**, 20170392. doi:10.1098/rsbl.2017.0392
- Richards, J. G. (2009). Chapter 10 metabolic and molecular responses of fish to hypoxia. *Fish Physiol.* **27**, 443–485. doi:10.1016/S1546-5098(08)00010-1
- Riesch, R., Oran, A., Dzienko, J., Karau, N., Schießl, A., Stadler, S., Wigh, A., Zimmer, C., Arias-Rodriguez, L., Schlupp, I. et al. (2010). Extreme habitats are not refuges: poeciliids suffer from increased aerial predation risk in sulphidic southern Mexican habitats. *Biol. J. Linn. Soc.* **101**, 417–426. doi:10.1111/j.1095-8312.2010.01522.x
- Rossi, G. S., Tunnah, L., Martin, K. E., Turko, A. J., Taylor, D. S., Currie, S., Wright, P. A. (2019). Mangrove fishes rely on emersion behavior and physiological tolerance to persist in sulfidic environments. *Physiol. Biochem. Zool.* **92**, 316–325. doi:10.1086/703117
- Sayer, M. D. J. (2005). Adaptations of amphibious fish for surviving life out of water. *Fish Fish* **6**, 186–211. doi:10.1111/j.1467-2979.2005.00193.x
- Schneider, S. M. (2016). Habitat choice and temporal variation alter the balance between adaptation by genetic differentiation, a jack-of-all-trades strategy, and phenotypic plasticity. *Am. Nat.* **187**, 633–646. doi:10.1086/685812
- Schneider, R. F. and Meyer, A. (2017). How plasticity, genetic assimilation and cryptic genetic variation may contribute to adaptive radiations. *Mol. Ecol.* **26**, 330–350. doi:10.1111/mec.13880
- Scott, G. R., Matey, V., Mendoza, J. A., Gilmour, K. M., Perry, S. F., Almeida-Val, V. M. F. and Val, A. L. (2017). Air breathing and aquatic gas exchange during hypoxia in armoured catfish. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **187**, 117–133. doi:10.1007/s00360-016-1024-y
- Tatarenkov, A., Ring, B. C., Elder, J. F., Bechler, D. L. and Avise, J. C. (2010). Genetic composition of laboratory stocks of the self-fertilizing fish *Kryptolebias marmoratus*: a valuable resource for experimental research. *PLoS One* **5**, e12863. doi:10.1371/journal.pone.0012863
- Taylor, D. S. (2012). Twenty-four years in the mud: what have we learned about the natural history and ecology of the mangrove rivulus, *Kryptolebias marmoratus*? *Integr. Comp. Biol.* **52**, 724–736. doi:10.1093/icb/ics062
- Tunnah, L., Robertson, C. E., Turko, A. J. and Wright, P. A. (2021). Acclimation to prolonged aquatic hypercarbia or air enhances hemoglobin–oxygen affinity in an amphibious fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **252**, 110848. doi:10.1016/j.cbpa.2020.110848
- Turko, A. J. and Rossi, G. S. (2022). Habitat choice promotes and constrains phenotypic plasticity. *Biol. Lett.* **18**, 20210468. doi:10.1098/rsbl.2021.0468
- Turko, A. J., Earley, R. L. and Wright, P. A. (2011). Behaviour drives morphology: voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus. *Anim. Behav.* **82**, 39–47. doi:10.1016/j.anbehav.2011.03.001
- Turko, A. J., Cooper, C. A. and Wright, P. A. (2012). Gill remodelling during terrestrial acclimation reduces aquatic respiratory function in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **215**, 3973–3980. doi:10.1242/jeb.074831
- Turko, A. J., Robertson, C. E., Bianchini, K., Freeman, M. and Wright, P. A. (2014). The amphibious fish *Kryptolebias marmoratus* uses different strategies to maintain oxygen delivery during aquatic hypoxia and air exposure. *J. Exp. Biol.* **217**, 3988–3995. doi:10.1242/jeb.110601
- Turko, A. J., Tatarenkov, A., Currie, S., Earley, R. L., Platek, A., Taylor, D. S. and Wright, P. A. (2018). Emersion behaviour underlies variation in gill morphology and aquatic respiratory function in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **221**, jeb168039. doi:10.1242/jeb.168039
- Turko, A. J., Maini, P., Wright, P. A. and Standen, E. M. (2019a). Gill remodelling during terrestrial acclimation in the amphibious fish *Polypterus senegalus*. *J. Morphol.* **280**, 329–338. doi:10.1002/jmor.20946
- Turko, A. J., Doherty, J. E., Yin-Liao, I., Levesque, K., Kruth, P., Holden, J. M., Earley, R. L. and Wright, P. A. (2019b). Prolonged survival out of water is linked to a slow pace of life in a self-fertilizing amphibious fish. *J. Exp. Biol.* **222**, jeb209270. doi:10.1242/jeb.209270
- Turko, A. J., Rossi, G. S. and Wright, P. A. (2021). More than breathing air: evolutionary drivers and physiological implications of an amphibious lifestyle in fishes. *Physiology* **36**, 307–314. doi:10.1152/physiol.00012.2021
- Urbina, M. A., Forster, M. E. and Glover, C. N. (2011). Leap of faith: voluntary emersion behaviour and physiological adaptations to aerial exposure in a non-aestivating freshwater fish in response to aquatic hypoxia. *Physiol. Behav.* **103**, 240–247. doi:10.1016/j.physbeh.2011.02.009
- Wells, M. W., Turko, A. J. and Wright, P. A. (2015). Fish embryos on land: terrestrial embryo deposition lowers oxygen uptake without altering growth or survival in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* **218**, 3249–3256. doi:10.1242/jeb.127399
- West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* **20**, 249–278. doi:10.1146/annurev.es.20.110189.001341
- West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. Oxford University Press.
- Wright, P. A. (2012). Environmental physiology of the mangrove rivulus, *Kryptolebias marmoratus*, a cutaneously breathing fish that survives for weeks out of water. *Integr. Comp. Biol.* **52**, 792–800. doi:10.1093/icb/ics091
- Wright, P. A. and Turko, A. J. (2016). Amphibious fishes: evolution and phenotypic plasticity. *J. Exp. Biol.* **219**, 2245–2259. doi:10.1242/jeb.126649

## **Table S1. Raw data**

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